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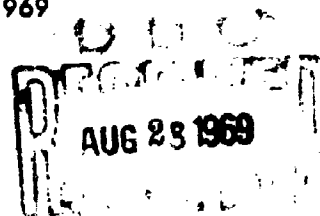
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TECHNICAL MANUSCRIPT 472

IDENTIFICATION OF CLONES OF  
MAMMALIAN CELLS BY ENZYMATIC ACTIVITY  
AND ISOENZYME DISTRIBUTION PATTERNS

Donald C. Fish  
James P. Dobbs  
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JULY 1969



DEPARTMENT OF THE ARMY  
Fort Detrick  
Frederick, Maryland

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IDENTIFICATION OF CLONES OF MAMMALIAN CELLS  
BY ENZYMIC ACTIVITY AND ISOENZYME DISTRIBUTION PATTERNS

Donald C. Fish

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Process Development Division  
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Project 1B562602A082

July 1969

ABSTRACT

Isoenzyme distribution patterns (zymograms) and enzymic-specific activities were determined for both suspension and monolayer cultures of the LM cell line, its clone (Cl 1), and four sub-clones from this clone. Identical zymograms were obtained from monolayer cultures of all clones for all enzymes tested. Suspension cultures of all clones yielded identical zymograms for six hydroxy acid dehydrogenases. However, when grown in suspension culture, reproducible and characteristic differences were found in the esterase zymograms from each clone. The growth pattern and esterase zymograms from sub-clone 1-9 varied more from those of the parent and the other sub-clones than any of the others. Thus, this procedure can be used to identify and characterize tissue cells and their clones in culture. The enzymic-specific activities from all cultures were identical when the cultures were sampled at the same stage in their growth cycle.

## I. INTRODUCTION\*

Since the initial observation about 10 years ago that different proteins with similar enzyme activity existed in the same cell, organism, or tissue,<sup>1-3</sup> the study of isoenzymes and their distribution patterns (zymograms) has expanded into almost every area of biological research. Zymograms have been used for the diagnosis of diseases, either human<sup>4</sup> or plant;<sup>5</sup> the identification and differentiation of bacterial<sup>6,7</sup> and animal<sup>1,2,8-13</sup> species; studies on developmental embryology;<sup>1,14</sup> the study of sex differences;<sup>15</sup> and for explaining control mechanisms in intermediary metabolism.<sup>16</sup> Perhaps the greatest future for this technique, though, is in its application to the study of genetic differences among cell lines<sup>17-19</sup> and the changes in genotype due to hybridization,<sup>20,21</sup> the presence of a sarcoma,<sup>22</sup> or virus infection.

Previous studies<sup>1,8-13,17,19,23</sup> were primarily concerned with zymogram differences between samples of the same tissue from different species, different tissues from the same species, or different cell lines. The work presented here describes how this technique may be utilized to study the differences among clones of the same cell line, so that identification of the clones, as well as environmental and nutritional effects upon genotypic and phenotypic expression, can be monitored.

## II. MATERIALS AND METHODS

### A. CELL STRAINS AND CLONING TECHNIQUE

Cultures of the LM cell line and a clone, Cl 1, from this cell line (after it had been cultured in a mouse and again isolated) were obtained from Dr. Donald Merchant. They were maintained serum- and antibiotic-free in spinner culture on medium 199 peptone (199P) by a series of half-splits for 30 months. Clone 1 was recloned by a one-step dilution method as follows: from the spinner, 10-μliter samples were removed and mixed with 10 ml of 199P containing 20% horse serum, 50 units penicillin per ml, and 50 μg streptomycin per ml. From this pool, 100 μliters of the cell suspension were placed in Falcon tissue-culture-grade plastic petri dishes containing 10 ml of the medium (199P, 20% horse serum, and antibiotics). The monolayer was incubated in a 5% carbon dioxide atmosphere. At 4 weeks, 15 to 35 colonies per petri dish, representing a 15 to 35% plating efficiency, were observed, and, from these, clones were picked at random.

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The clones were first isolated with glass tubing (6 mm) wells, then aspirated from the surface of the petri dish with fresh cloning medium, and placed as monolayers in Falcon T-30 flasks. Cells from the monolayer cultures of the individual clones were harvested, resuspended at a concentration of about  $1 \times 10^7$  cells/ml in fresh medium containing 5% dimethyl sulfoxide, and stored at liquid nitrogen temperatures.

#### B. GROWTH OF CELLS AND PREPARATION OF CELL-FREE EXTRACTS

The clones were removed from the freezer and grown in 199P with no serum or antibiotics, either as monolayers or in suspension culture. Cultures were obtained by placing the contents (1 ml containing  $1 \times 10^7$  cells) of a frozen ampule in 50 ml of medium. This was used to seed either monolayer (25 ml per T-60 flask) or suspension (50 ml per 200-ml spinner flask; added 0.12% methylcellulose,\* 15 CPS) cultures. Monolayer cultures were grown at 37 C for 3 to 4 days until a confluent monolayer formed. Suspension cultures were grown at 37 C for 5 to 6 days until the count reached  $1.5 \times 10^6$  to  $2.0 \times 10^6$  cells/ml.

The cells were scraped from the glass with rubber policemen, sedimented by centrifugation (10 minutes at 500 x g), washed once with saline, resuspended in 1.0 to 2.0 ml water, and then stored at -20 C until disrupted. Suspension cultures were centrifuged and processed in the same fashion. Loss of enzymic activity was less in cells stored this way than in stored cell-free extracts. However, under no conditions did storage exceed 1 month.

Cell-free extracts were prepared both by a three-cycle freeze-thaw procedure and by sonic oscillation. Both procedures resulted in preparations of approximately equal activity, but for ease in handling and time involved we decided to use sonic oscillation. The cells were thawed and the liquid volume was adjusted to 1.0 or 2.0 ml with water, depending upon the cell mass available. Water, instead of buffer, was used to dilute the cells because of the greatly superior results reported by Paul and Fottrell<sup>12</sup> in similar studies. A Branson sonifier,\*\* model W, was used at setting #1 (15 watts) for 30 second while the centrifuge tube containing the cells was immersed in a 4 C water bath. The sample was frozen and thawed, and the supernatant liquid, after a single centrifugation at 1,000 x g for 10 minutes, was used for enzymic assay and disc electrophoresis. This freeze-thaw cycle yielded clearer supernatant liquids than those obtained from a direct centrifugation following sonic disruption.

Protein concentration was determined spectrophotometrically by the difference in absorption at 215-225 m $\mu$ .<sup>24</sup>

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\* Fisher Scientific Co., Silver Spring, Md.

\*\* Branson Instruments Co., Stamford, Conn.

### C. DISC ELECTROPHORESIS

Polyacrylamide gel disc electrophoresis was performed by the technique of Ornstein<sup>25</sup> and Davis<sup>26</sup> as described by Canal Industrial Corp. The tubes, 6 mm I.D. by 75 mm, were not fire-polished and were coated with 1% column coat prior to use to facilitate removal of the gel. Acrylamide was recrystallized from chloroform (50 g/liter) at 50 C. Bis-acrylamide was recrystallized from acetone (10 g/liter) at 45 C.

The separating gel was made by mixing one part A\* (with twice the indicated concentration of T-med), one part water, two parts C (acrylamide 34 g, bis 0.85 g), and four parts G. This gel was 55 mm long. The spacer gel, one part B, two parts D, two parts F, and three parts G in 0.15-ml amounts, was placed on top of the separating gel after the latter had polymerized and the water (necessary in order to obtain a flat surface) had been removed.

Samples were diluted in 50% sucrose and had a final sucrose concentration of at least 15%. All gels were run at a protein concentration of 0.2 mg/gel. The sample, 0.1-ml volume, was placed on top of the polymerized spacer gel. A thin cap gel (spacer gel) was added and buffer was placed on top of this.

The gels were developed either at constant current (50 ma/24 gels) or constant voltage (80 V/24 gels). The bromphenol blue tracking dye migrated almost to the end of the separating gel in 2.5 to 3 hours, depending upon the age of the buffer (solution H). The gels were flushed from the tubes by a fine stream of water and cut at the location of the tracking dye so that band position could be determined by  $R_f$  value. The gels were washed for 5 minutes in cold 0.02 M tris-HCl buffer, pH 7.1, and then placed in the various staining solutions in a 37 C water bath. Precautions were taken to avoid exposing staining solutions to light.

### D. ISOENZYME STAINING PROCEDURES

Esterase substrates studied were alpha-naphthyl acetate, alpha-naphthyl propionate, alpha-naphthyl butyrate, alpha-naphthyl caproate, and beta-naphthyl acetate (1 g/100 ml acetone). Dye-substrate mixtures (5 ml/gel) were prepared by combining 150  $\mu$ moles tris-HCl buffer, pH 7.1, 0.12 ml substrate, and 2.6 mg fast blue RR. Lactic and malic dehydrogenase activities were determined in a solution containing 100  $\mu$ moles tris-HCl buffer, pH 7.1; 70  $\mu$ moles KCN, pH 7.5; 500  $\mu$ moles substrate; 1.5 mg nicotinamide adenine dinucleotide (NAD); 1.5 mg nitro blue tetrazolium (NBT); and 0.07 mg "phenazine methosulfate" (PMS). Malic dehydrogenase was determined with both NAD and nicotinamide adenine dinucleotide phosphate (NADP) as cofactors.

\* Components as described in Ornstein<sup>25</sup> and Davis.<sup>26</sup>



Glucose-6-phosphate dehydrogenase activity was determined in a solution containing 3  $\mu$ moles  $\text{MnSO}_4$ ; 1,700  $\mu$ moles tris-HCl buffer, pH 7.1; 7 mg glucose-6-phosphate; 2 mg NADP; 2 mg NBT; and 0.1 mg PMS. D,L-Isocitric dehydrogenase activity was determined in a solution containing 217  $\mu$ moles tris-HCl buffer, pH 8.0; 7 mg D,L-isocitric acid; 2 mg NADP; 2 mg NBT; and 0.1 mg PMS. Alpha-hydroxy acid oxidase activity was determined by a modification of the method of Allen and Beard.<sup>27</sup> The reaction mixture contained 200  $\mu$ moles potassium phosphate buffer, pH 7.5; 500  $\mu$ moles substrate; 2 mg NBT; 2 mg NAD; and 0.1 mg PMS. L-alpha-glycerophosphate dehydrogenase activity was determined by the procedure of Fiala.<sup>28</sup> Protein was stained for 1 hour in a solution of 2 g amido schwarz/100 ml methanol:water:acetic acid (5:5:1) and then destained in several changes of methanol:water:acetic acid (4:10:1).

After staining was completed, the stain solution was decanted and replaced by an equal volume mixture of ethanol and 10% acetic acid.<sup>29</sup> The gels were fixed for several hours and then stored in 10% acetic acid in stoppered test tubes.

#### E. ENZYMIC-SPECIFIC ACTIVITY

The specific activities of ten enzymes were determined by the procedures outlined by Daniels et al.\* The specific activities of acid and alkaline phosphatase, amino peptidase, and lipase are reported as optical density units per milligram protein. All the others are reported as units per milligram protein, using an extinction coefficient of  $6.22 \times 10^6 \text{ cm}^2/\text{mole}$  for the reduced cofactor.

### III. RESULTS

#### A. GROWTH CHARACTERISTICS OF VARIOUS CLONES

All monolayers were inoculated at the same cell concentration ( $4 \times 10^5$  cells/ml) and harvested at the same phase of growth (just as they formed a confluent monolayer). Confluent monolayers were obtained after 3 to 4 days' incubation from every clone except Cl 1-9, which required 6 to 7 days. Cells from Cl 1-9 appeared larger than cells from the other clones and remained more spread out or amoeboid, and the monolayers never became as dense as those obtained from the other clones.

Suspension cultures were maintained by half-splits in the late log period of growth. All clones except Cl 1-9 were split every 3 to 4 days; Cl 1-9 required 6 to 7 days growth. Even then, the cell density (Table 1) never reached the values obtained from the other clones.

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\* Daniels, W.F.; Fish, D.C.; Garcia, L.; Rosensteel, J.; Dobbs, J.P.  
Unpublished data.

TABLE 1. COMPARISON OF MAXIMUM CELL DENSITY OBTAINED IN  
SUSPENSION CULTURES OF THE PARENT AND ITS CLONES

Cell Line	Replicates	Mean Maximum Cell Density <sup>a</sup> / $\times 10^6$	Standard Deviation
LM	8	1.62	0.24
Cl 1	4	1.55	0.44
Cl 1-1	9	1.42	0.27
Cl 1-2	4	1.75	0.77
Cl 1-3	7	1.45	0.24
Cl 1-9	5	0.69	0.05

a. Viability in all cultures exceeded 93%.

#### B. EFFECTS OF MINOR VARIATIONS IN PROCEDURE

Identical patterns were obtained when a large batch of cells was disrupted by sonic oscillation and the cell-free extract was divided into three equal portions and subjected to disc electrophoresis on days 1, 3, and 7 after sonication and storage at -20 C.

For optimum esterase activity, the pre-staining rinse in tris-HCl buffer was required. Esterase activity was inferior when tris-maleate buffer was substituted for the tris-HCl buffer.

Faster migration rates but hazier bands were obtained both with esterase (alpha-naphthyl acetate) and lactic dehydrogenase (Fig. 1) when the buffer concentration used to make the gels was decreased from 3.0 M to 0.5 M and the gel concentration was decreased from 8.5% to 7.0%. The highest gel and buffer concentrations are shown as A and the lowest concentrations as B. This effect was magnified when the gel concentration was lowered to 5.5%. The loss of resolution in the 0.5 M buffer - 7.0 or 5.5% gel system was so great that its use was discontinued. The T-med concentration was varied between 0.18 and 0.46 ml/100 ml, with the highest concentration yielding the best results. The lack of a spacer gel (D compared with C) resulted in more rapid migration and consequently less distinct bands (Fig. 1). Omission of the cap gel on top of the sample also resulted in poorer quality of gels.

Although the protein concentration was adjusted to 0.2 mg/gel in all studies on comparing the different clones, varying the protein concentration between 0.2 and 0.8 mg/gel had no effect upon the number, position, or relative intensity of the bands (Fig. 2). Of the four stains commonly used for protein, Coomassie blue,<sup>30</sup> Procion blue,<sup>31</sup> and 2,2-dihydroxy-6,6'-dinaphthyl disulfide (0.5% in 7.5% acetic acid) did not consistently yield as good results as did the amido schwarz.

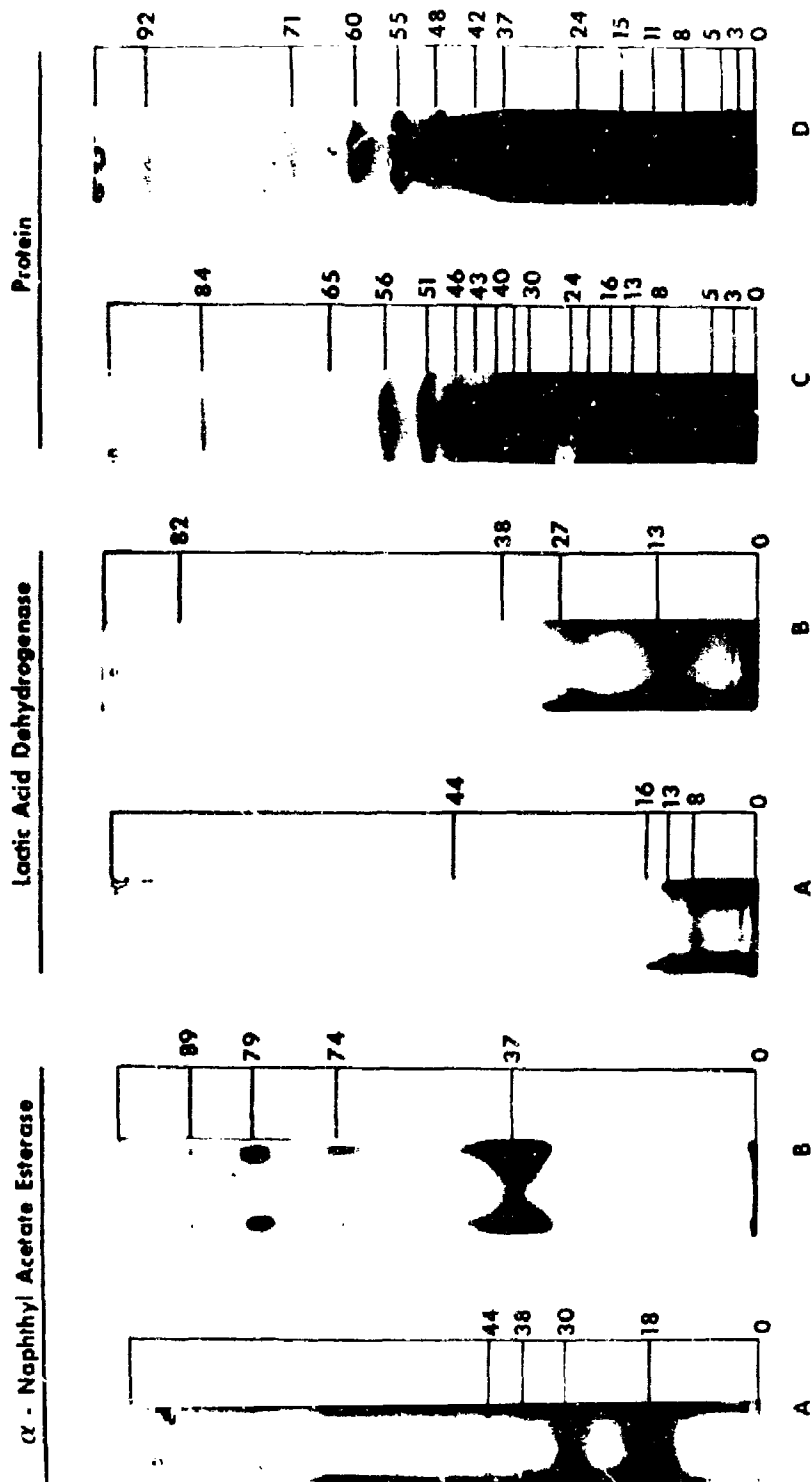


FIGURE 1. Effect of Gel and Buffer Concentration and Presence or Absence of Spacer Gel on Zymograms.  
 A, 8.5% gel and 3.0 M buffer concentration; B, 7.0% gel and 0.5 M buffer concentration; C, presence of spacer gel; D, absence of spacer gel.

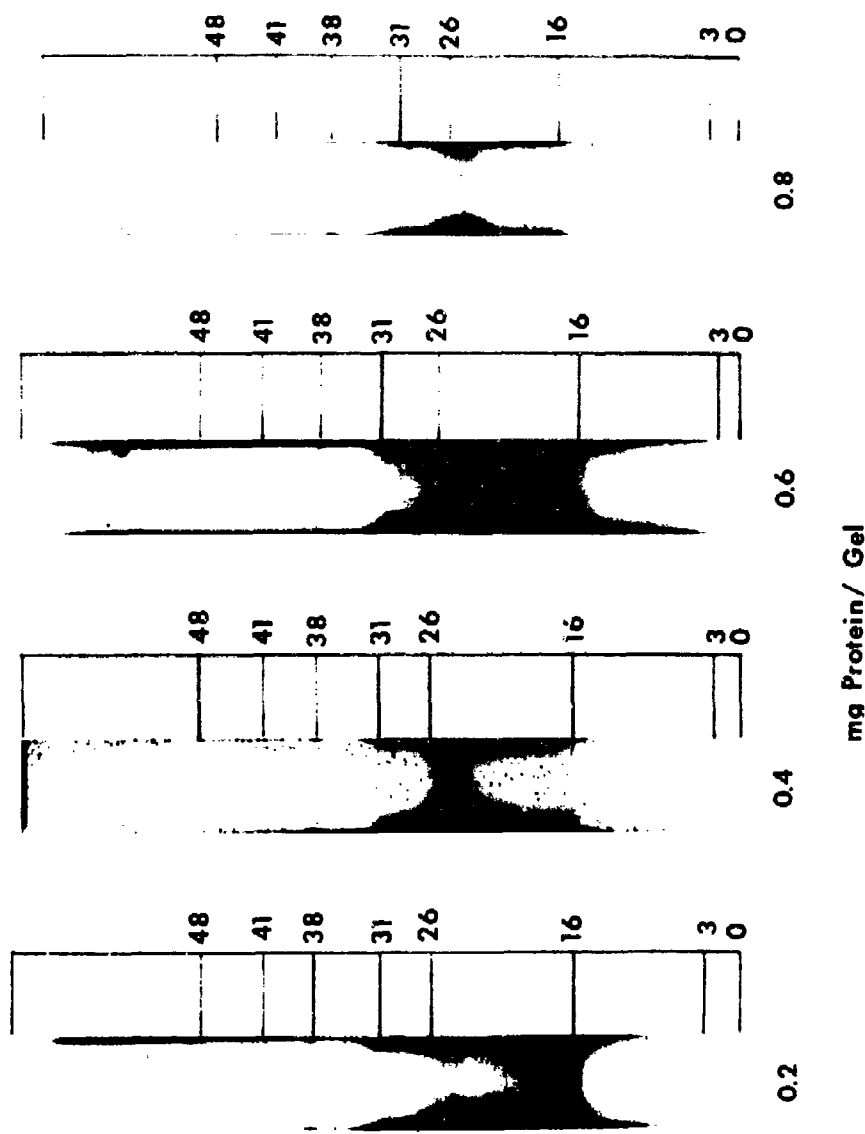


FIGURE 2. Effect of Protein Concentration on Beta-Naphthyl Acetate Esterase Activity.

In the absence of substrate (alpha-naphthyl acetate or lactic acid) no bands appeared. In an attempt to see if bands were being lost by virtue of their migration to the cathode rather than the anode,<sup>22</sup> the electrodes were reversed in several experiments and the gels were stained for protein, lactic dehydrogenase, or alpha-naphthyl propionate esterase activity. No bands were detected in the gels by the above methods.

Contrary to the results of enzyme assay on the supernatant liquids or of other reports in the literature, we were unable to demonstrate any of the following enzyme activities following disc electrophoresis: amino peptidase; acid and alkaline phosphatase; beta-naphthyl laureate, alpha-naphthyl stearate, and alpha-naphthyl caprylate esterase; cholinesterase; succinic, ethanol, isopropanol, isoamyl, glutamic, propionaldehyde, benzaldehyde, formaldehyde, D,L-glyceraldehyde, acetaldehyde, sorbitol, choline, glucose, alpha-keto-glutarate, 6-phosphogluconate, and D-glyceraldehyde-3-phosphate dehydrogenase; hexokinase; amylase; peroxidase, catalase; beta-glucuronidase; or glutamic/oxalacetic and glutamic/pyruvic transaminase. Most, but not all, of these procedures were run simultaneously on rat serum, kidney, or liver extracts to provide positive controls.

No selective effect on the various esterase isoenzyme bands was noticed in the presence of  $\text{CaCl}_2$ ,  $\text{CoCl}_2$ ,  $\text{MnSO}_4$ ,  $\text{MgSO}_4$ , ethylenediaminetetraacetate (EDTA), eserine, para-hydroxy(chloro)mercuribenzoate, or alpha, alpha'-dipyridyl. Optimum activity was found in the presence of EDTA (0.05 M), but cysteine-HCl (0.1 M) completely inhibited all activity. Neither zinc acetate nor EDTA affected the NAD-linked malic dehydrogenase activity.

The isoenzyme distribution pattern for lactic dehydrogenase and alpha-naphthyl acetate and propionate esterase was not altered by sonication at setting #1 for 15 seconds to 5 minutes and at settings #3 and #5 for 1 minute.

#### C. ISOENZYME DISTRIBUTION PATTERNS OF CLONES GROWN IN SUSPENSION CULTURE

Each clone as well as the parent cell lines gave a reproducible and characteristic zymogram for esterase activity (Fig. 3 and 4 and Table 2). The original cell line was labelled LM. This cell line was injected into mice, reisolated, and cloned to yield the parent Cl 1. This parent was then recloned to yield Cl 1-1, 1-2, 1-3, and 1-9. Although the results from monolayer cultures are shown in the figures according to their respective substrates, these results will be formally presented below. The longer the ester-linked side chain became, the lower was the total relative esterase activity. Consequently, the incubation times had to be adjusted; however, no new bands appeared upon prolonged incubation.

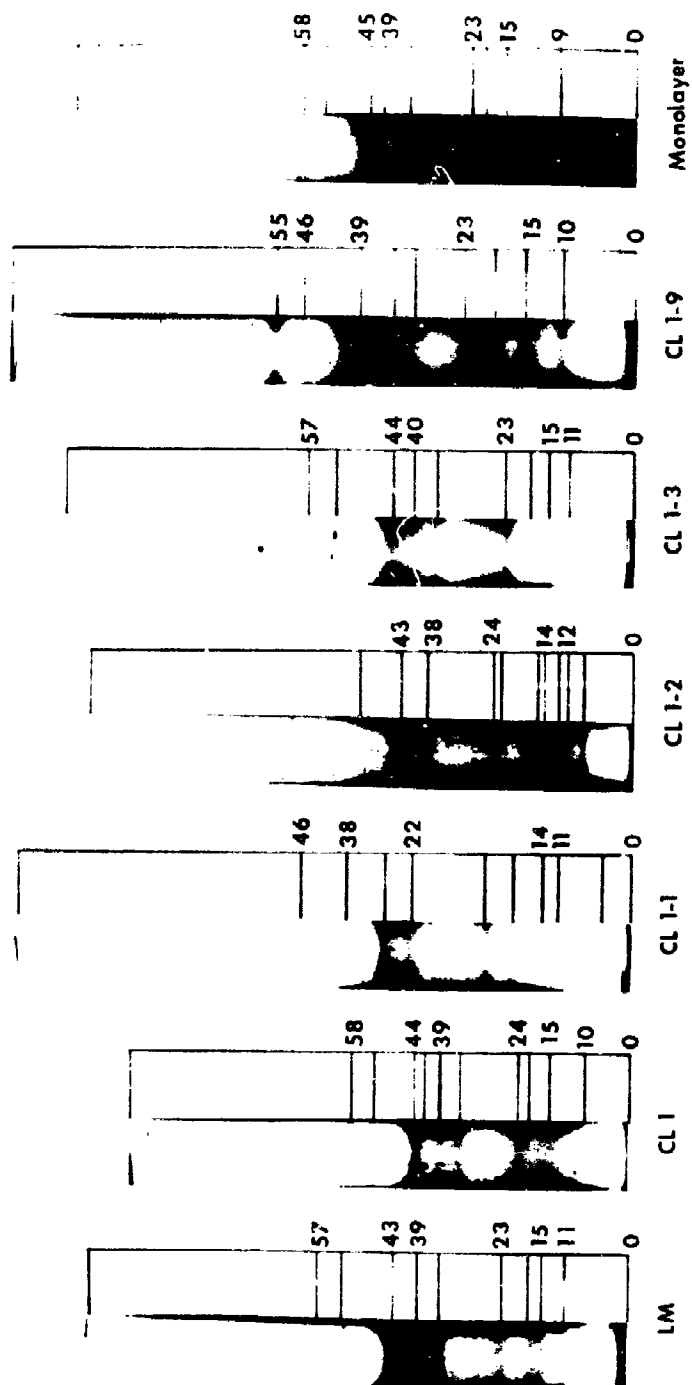


FIGURE 3. Characteristic Zymogram for Alpha-Naphthyl Propionate Esterase from the Various Clones.

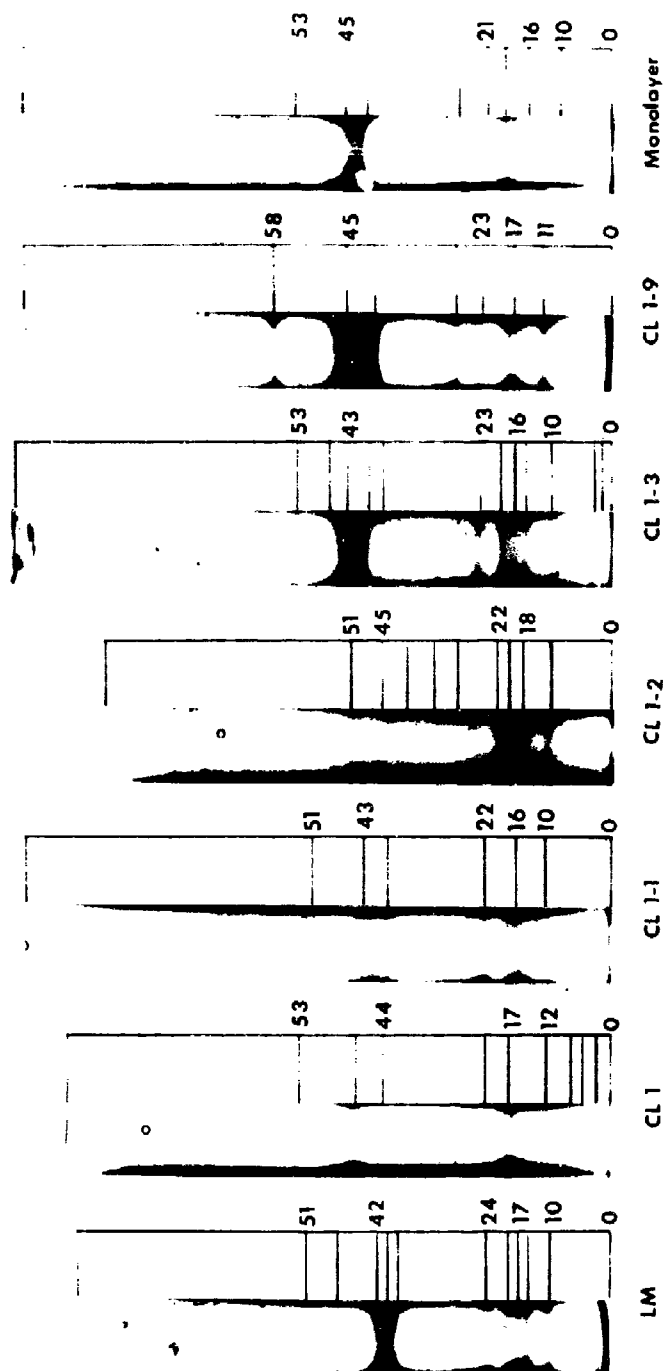


FIGURE 4. Characteristic Zymogram for Alpha-Naphthyl Butyrate Esterase from the Various Clones.

TABLE 2.  $R_f$  VALUES FOR ISOENZYMES FROM THE VARIOUS CELL CULTURES

Enzyme Substrate		$R_f$ Values for Isoenzyme Bands															
Alpha-Naphthyl Acetate																	
LM	0	12	15	19	21	24	29	31	34	39	42	49	51	59	64		
Cl 1	0	4	10	15		24		31	35	38	44		52	58			
Cl 1-1	0					23		31	34	38	41		49	56			
Cl 1-2	0						27	31	35	40		46	48	54			
Cl 1-3	0		12	14			27	31	35	39	42		49	57			
Cl 1-9	0	3	5	11	15		24	28	31	38	42		48	55	60		
Monolayer	0				22	26	29		33	38	43		48	57			
Alpha-Naphthyl Propionate																	
LM	0	11	15	21	23		35	39	43		52	57					
Cl 1	0	10	15	21	24		33	39	42		52	58					
Cl 1-1	0	3	11	14	16		36	38	46								
Cl 1-2	0	9	12	14	17	19	26	38	43	48							
Cl 1-3	0		11	15	17	23	34	40	44		52	57					
Cl 1-9	0		10	15	18	23	36	39	46		55						
Monolayer	0	9	15	19	23	28		39	45		52	58					
Alpha-Naphthyl Butyrate																	
LM	0		10	15	17	19	24		39	41	42	47	51				
Cl 1	0	3	5	7	12	17	19				44	49	53				
Cl 1-1	0			10	16	18	22		38		45	51					
Cl 1-2	0			13	18	20	22	27	31	42	45	51					
Cl 1-3	0	2	3	10	15	16	18	23	39	41	43	46	53				
Cl 1-9	0			11	17	17	23	27		42	45	58					
Monolayer	0		10	10	16	18	21	25		41	45						
Beta-Naphthyl Acetate																	
LM	0	12	15	19	22	27	38	43	49	57							
Cl 1	0	6	15	21	23	25	34	43									
Cl 1-1	0			23	26	29	36	40	54								
Cl 1-2	0	3	5	22	24	25				56	59	64	73				
Cl 1-3	0			23	25	34	39	44									
Cl 1-9	0		11	22	22	32	38	42	48	56		61					
Monolayer	0	12	17	23	27	32	38	42	57								
Protein																	
Others	0	2	3	7	8	10	15	18	19	22	26	31	35	38	43	50	53
Cl 1-9	0	2	3	5	7	10	12	15	18	20	23	27	-	-	-	49	54
Monolayer	0	2	3	5	7	9	10	13	15	17	18	20	22	27	28	32	35



None of the clones showed any difference in hydroxy acid dehydrogenase activity, and all patterns resembled that obtained for lactic dehydrogenase (Fig. 5). As was true with esterase activity, the relative enzymic activity decreased as chain length increased. This is reflected both in the number and intensity of the isoenzyme bands visualized.

The protein patterns from all clones except Cl 1-9 were identical with each other and with that of the parent lines (LM and Cl 1). Cl 1-9 differed in that it possessed one extra band and was missing five bands compared with the other cultures.

These patterns were reproducible in duplicate runs on the same sample and remained constant during continuous subculture over a 2-month period. One of the clones, Cl 1-1, has been maintained continuously for 1 year with no observable changes in pattern.

#### D. ISOENZYME DISTRIBUTION PATTERNS OF CLONES GROWN IN MONOLAYER CULTURE

In contrast to the results from the various clones and cell lines grown in suspension culture, there were no detectable differences in any of the zymograms from the same cells grown in monolayer cultures. However, the zymogram from monolayer cultures was quite different from that obtained from suspension cultures. Representative gel patterns are included for each stain in Figures 3, 4, and 6 and Table 2. These patterns were both reproducible and constant through consecutive subculture over a 6-month period.

#### E. MISCELLANEOUS COMPARISONS OF ZYMOGRAMS

The zymogram for alpha-glycerophosphate dehydrogenase (Fig. 7) was identical for all clones grown in suspension culture. Its activity was not determined from monolayer cultures. The zymogram for glucose-6-phosphate dehydrogenase (Fig. 7) was identical for all clones grown in both suspension and monolayer cultures. The zymograms for alpha-naphthyl caproate esterase (Fig. 7) and malic dehydrogenase (Fig. 8) did not reveal any differences among the various clones but the latter did show marked differences related to the cofactor used.

#### F. ENZYMIC SPECIFIC ACTIVITY

The mean value and average standard deviation of the mean for the specific activity of ten enzymes from the cells of the various clones grown both in suspension and monolayer cultures are given in Table 3. With the possible exception of hexokinase activity in Cl 1-3 (suspension) and alkaline phosphatase activity in Cl 1-1 (monolayer), none of the specific activities appears to show any characteristic clonal differences.

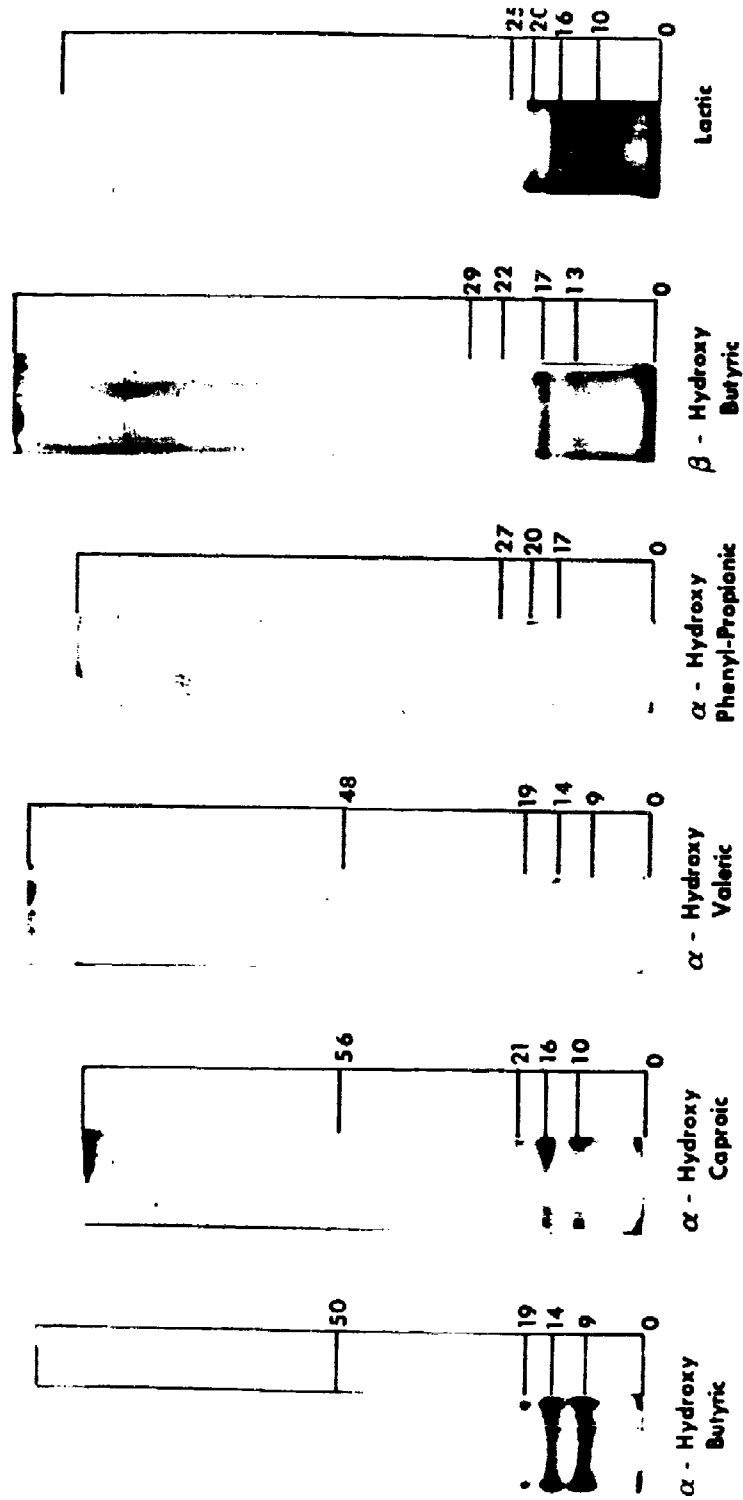


FIGURE 5. Representative Zymograms of all Suspension Cultures on Six Different Hydroxy Acids.

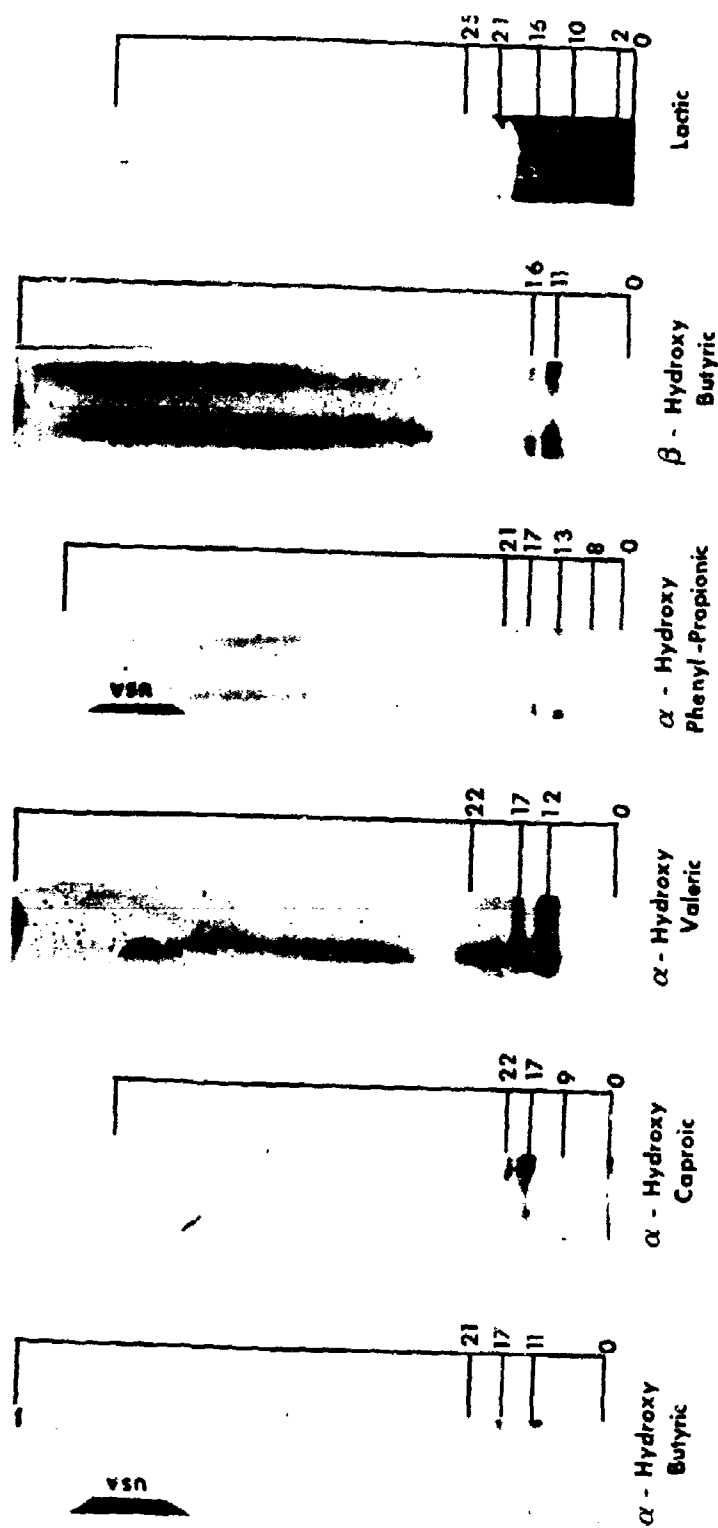


FIGURE 6. Representative Zymograms of all Monolayer Cultures on Six Different Hydroxy Acids.

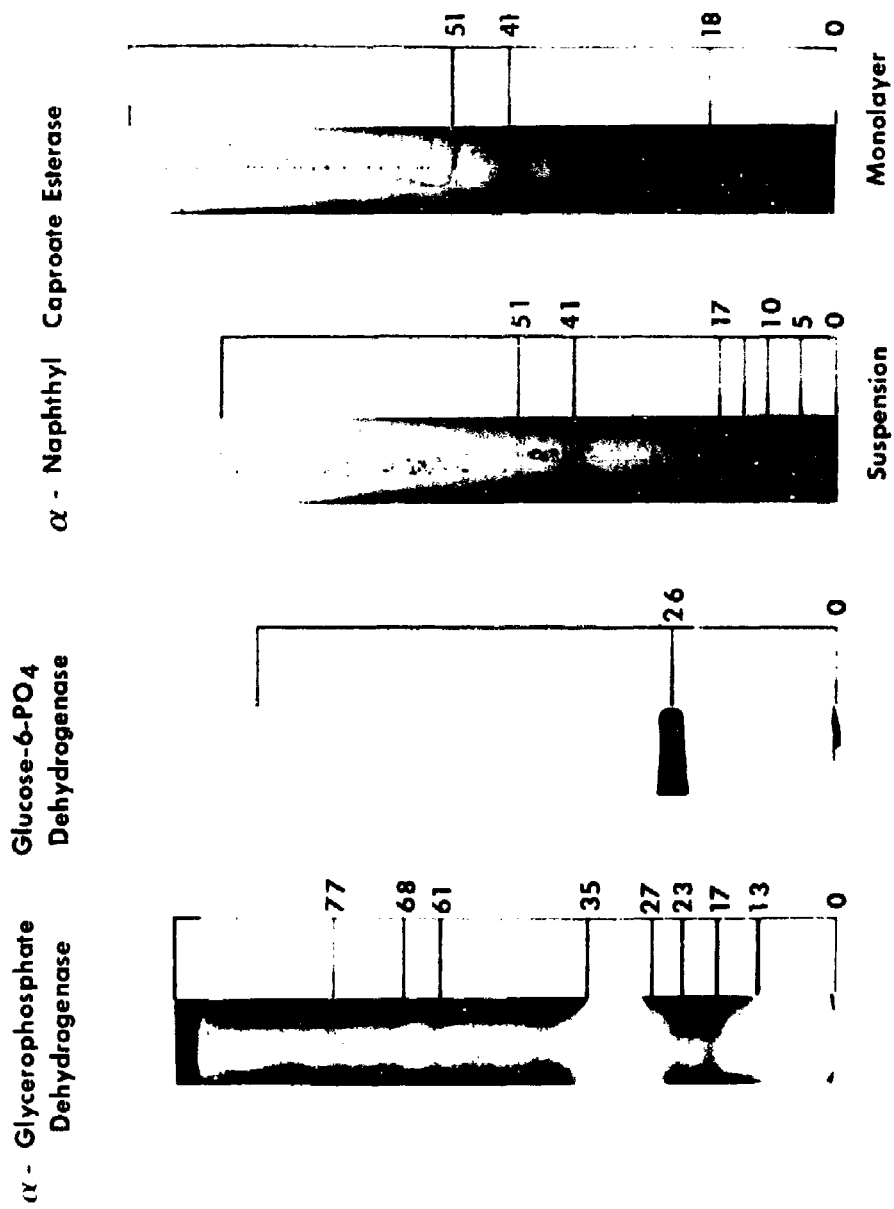


FIGURE 7. Miscellaneous Zymogram Comparisons.

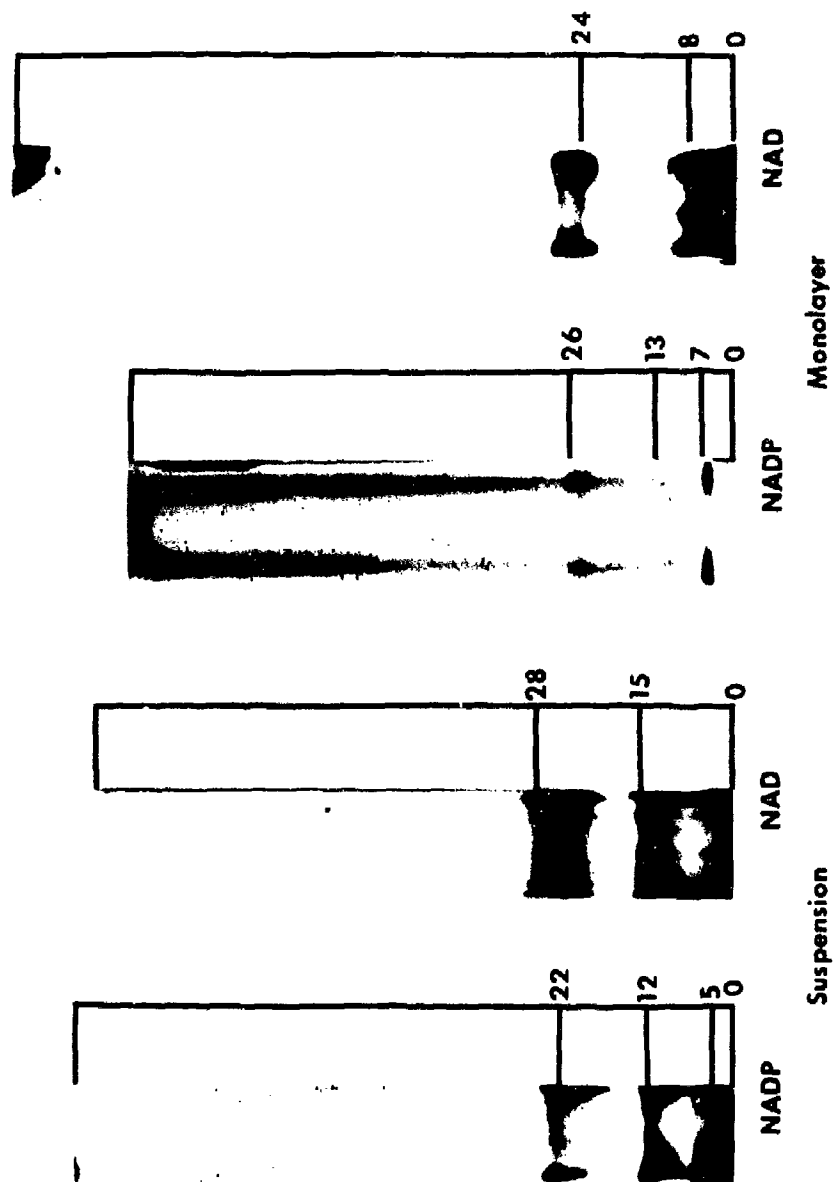


FIGURE 8. Effect of Cofactor Used on Malic Dehydrogenase Zymograms.

TABLE 3. MEAN ENZYMIC SPECIFIC ACTIVITY<sup>a</sup>/ FROM CELLS GROWN IN SUSPENSION OR MONOLAYER CULTURE

Enzyme Activity	Cell Lines and Clones						SD <sup>b</sup>
	LM	Cl 1	Cl 1-1	Cl 1-2	Cl 1-3	Cl 1-9	
<u>Suspension Culture</u>							
D, L-Isocitric dehydrogenase	0.091	0.098	0.098	0.095	0.125	0.154	0.005
Lactic dehydrogenase	2.91	2.41	2.95	1.98	2.48	2.56	0.34
Malic dehydrogenase	3.02	2.34	3.18	1.93	2.44	2.26	0.31
Glucose-6-phosphate dehydrogenase	0.115	0.126	0.108	0.127	0.139	0.140	0.009
Hexokinase	0.0051	0.0100	0.0067	0.0076	0.0216	0.0058	0.0018
D, L-Glyceraldehyde-3-phosphate dehydrogenase	0.038	-	0.041	-	0.040	0.049	0.005
Alkaline phosphatase	1.75	-	1.74	-	1.60	1.70	0.16
Acid phosphatase	5.92	4.58	6.04	4.68	6.14	6.54	0.62
Amino peptidase	2.27	1.76	2.22	1.73	2.34	2.65	0.20
Lipase	0.96	0.70	0.61	0.52	0.79	1.17	0.14
<u>Monolayer Culture</u>							
D, L-Isocitric dehydrogenase	0.110	0.078	0.185	0.070	0.158	0.130	0.009
Lactic dehydrogenase	2.34	1.40	3.78	-	1.76	-	-
Malic dehydrogenase	1.69	-	4.47	-	2.31	1.49	-
Glucose-6-phosphate dehydrogenase	0.217	0.124	0.206	0.130	0.201	0.263	0.018
Hexokinase	0.0138	0.0065	0.0107	0.0064	0.0185	0.0203	0.0023
D, L-Glyceraldehyde-3-phosphate dehydrogenase	0.035	-	0.062	-	0.033	0.029	0.012
Alkaline phosphatase	1.62	0.96	3.26	0.49	1.62	1.39	0.15
Acid phosphatase	4.45	4.29	4.21	3.30	4.12	3.91	0.59
Amino peptidase	2.50	1.14	3.24	0.87	2.20	2.16	0.14
Lipase	0.88	0.89	0.97	0.81	1.10	1.27	0.10

a. The means are based on two to 10 replicate samples (in most cases six to nine) per value.

b. This is the average standard deviation of the mean values for each of the five clones.

#### IV. DISCUSSION

Because polyacrylamide gel disc electrophoresis is still a relatively new technique as applied to tissue culture cells, the following control experiments were performed before interpreting the results obtained from the various clones. Many of the observations of Dietz and Lubrano<sup>33</sup> were confirmed in that (i) rate of migration increased when the spacer gel was omitted or when gel concentration or ionic strength of the buffer incorporated into the gel was decreased, (ii) duplicate samples yielded reproducible zymograms, (iii) samples, provided that the protein concentration was greater than 1 to 2 mg/ml, could be stored at -20 C for several weeks and still yield reproducible zymograms, (iv) increasing sample concentration merely decreased staining time and did not change either the relative intensity of the various bands or result in the formation of new bands, and (v)  $R_f$  values, using the tracking dye front, were quite adequate and reproducible for identifying the various isoenzymes.

However, we did observe several important differences between our work and theirs and, since the methods differed essentially only in the concentration of gel and T-med, these results probably reflect fundamental differences between the types of sample used (human serum versus tissue cell extracts). Our patterns were clearer and more reproducible when a stacking gel was present and a pre-stain wash included. Moreover, a more concentrated gel (8.5%) gave much better zymograms than a dilute gel (7.0 or 5.5%) with our samples. Although both samples had five bands of lactic dehydrogenase, the migration rates were quite different. The lactic dehydrogenase isoenzyme located at the juncture of the stacking and separating gel did not appear to be a "nothing" dehydrogenase<sup>33-35</sup> because, in the absence of substrate, no band forms in a comparable time period. However, following overnight incubation in the absence of substrate there was slight formation of a band at the interface.

When the gels were stained for comparable time periods in the absence of substrate no bands were noticed. When the substrate-less controls were allowed to stain overnight, some indication of a band at the interface was noticed. The activity at the interface as well as the wide and jagged bands noted with NAD-linked malic dehydrogenase (Fig. 8) are probably due to proteins that are too large to enter the pores of the gel and so lie at the interface or are forced to penetrate between the gel and the wall of the glass tube.<sup>28</sup> In many of the dehydrogenase zymograms (Fig. 5 and 6) and especially in those in which enzymic activity was low and consequently incubation time was long, characteristic negative bands were observed. These bands have been shown to be due to the presence of excess PMS.<sup>36</sup> Control experiments further showed that no bands were being lost due to migration to the cathode under the conditions of the experiment.

Esterase isoenzymes have been classified on the basis of their relative activity against various substrates<sup>11,37</sup> and sensitivity to various inhibitors or activators.<sup>37,38</sup> In contrast to these previous reports, which indicate the presence of a variety of classes of esterases in cell extracts, all our esterase isoenzymes appeared to be arylesterases,<sup>37</sup> because the reaction rate decreased as the length of the side chain increased and as the naphthyl linkage changed from alpha to beta. Markert and Moller<sup>1</sup> proposed that all alpha-hydroxy acids were oxidized by the same dehydrogenase and the zymograms shown in Figures 5 and 6 appear to support this conclusion. As was the case with the esterases, activity decreased as chain length increased.

Certain enzyme activities [hexokinase, D-glyceraldehyde-3-phosphate dehydrogenase, amino peptidase, acid and alkaline phosphatase, and lipase (beta-naphthyl laureate)] could be demonstrated colorimetrically in the cell-free extracts but could not be detected by the zymogram method. This could be due to (i) inactivation of the enzyme by the ammonium persulfate catalyst,<sup>39,40</sup> (ii) the enzyme's being too large to penetrate the pores of the gel, or (iii) the concentration of the enzyme being too low to be detected in the gels.<sup>17,41,42</sup>

Different isoenzymes have been shown to have different susceptibilities to sonic oscillation.<sup>43</sup> However, these effects were noticeable only after relatively long time periods (5 minutes) and at rather high power levels.<sup>6</sup> Neither the work of Regan<sup>44</sup> nor the work reported here indicated a change in zymogram as a result of varying the time of sonication from 15 seconds to 5 minutes. The time and intensity used in these studies were much lower than those used by Dubbs<sup>43</sup> in his study. However, microscopic examination indicated that these mild conditions of sonication were sufficient to disrupt essentially all of the cells.

The zymograms of alpha-hydroxy acid dehydrogenases from the clones grown both in suspension (Fig. 5) and in monolayer (Fig. 6) were identical for all clones tested and for all substrates. The change in pattern with increasing chain length of the alpha-hydroxy acid reflects a change in a catalytic ability of the various isoenzymes. However, no characteristic differences were noticed among the clones themselves when grown either in suspension or monolayer. The malic dehydrogenase zymograms (Fig. 8) did not indicate any differences among the various clones. However, the patterns were quite distinct from the lactic dehydrogenase zymograms and the marked effect of a different cofactor (indicating different isoenzymes) is readily apparent.

Although the clones all possessed identical alpha-hydroxy acid zymograms they could be readily distinguished by their esterase zymograms (Fig. 3 and 4 and Table 2). The differences in zymograms among the various clones are clearly evident and these patterns are reproducible for at least 2 to 6 months of consecutive subculture. Cl 1-9, which shows the greatest difference in growth characteristics from the parent and from the other



clones (Table 1), has the most markedly different zymogram. In fact, Cl 1-9 differs so much from the others that its protein pattern is different. The other clones cannot be distinguished from each other or from the parent on the basis of their protein patterns.

The zymograms from these same clones and from parent and grandparent when grown in monolayer all exhibited the same pattern (Fig. 3, 4, and 6 and Table 2). Thus, although differences among the clones could be distinguished following growth in suspension, differences could not be distinguished following growth as monolayers. The equilibrium between the zymogram characteristic of suspension culture and that found in monolayer culture is rapidly attained. Monolayers were seeded from suspension cultures of each clone and when harvested and tested at the end of the first passage the monolayers all yielded identical, typical "monolayer-type" zymograms. The figures show that the zymograms from cells grown in monolayer are different from those of the same clones grown in suspension and probably reflect a rather fundamental difference in the cells grown under these two different conditions.

DeLuca and colleagues<sup>45-47</sup> and others<sup>42,48,49,\*</sup> have studied changes in the specific activity of a variety of enzymes under various cultural conditions or with various cell lines. The specific activity of several enzymes not only appears to be a useful and reproducible characteristic of the particular cell line but the fact that it changes as a function of the growth cycle provides a useful method for determining if replicate cultures are in the same phase of growth. That the enzymic-specific activities of the various clones grown in both monolayer and suspension are similar (Table 3) provides further proof that the differences in zymogram are due to differences among the clones and do not reflect differences due to the cells' being in various stages of the growth cycle.

DeLuca<sup>46</sup> found that lactic dehydrogenase and glutamic-oxalacetic transaminase specific activity from the same cell lines grown either in suspension or monolayer never reached similar values, but that observed for glucose-6-phosphate dehydrogenase from the two different culture methods did as the cells approached the plateau phase. Our observations (Table 3) that specific activities in the same cell line or clone at the same phase in the growth cycle are similar under both conditions of growth, with the possible exception of hexokinase (three- to fourfold greater in monolayers), indicated that each cell line reacts differently to changes in its mode of culture.

Gartler<sup>18</sup> surveyed 20 heteroploid human cell lines and found identical zymograms for glucose-6-phosphate dehydrogenase and phosphoglucomutase.

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\* Daniels, W.F.; Fish, D.C.; Garcia, L.; Rosensteel, J.; Dobbs, J.P.  
Unpublished data.

After showing that changes did not occur as a result of in vitro culturing, he concluded that all the cultures had been contaminated by HeLa cells. Our work with L cell clones shows no differences among many of the dehydrogenase zymograms but recognizable and reproducible differences among the esterase zymograms. In view of this, an alternative interpretation to include the possibility that certain enzymes are more prone to phenotypic and/or genotypic variation than others might be more meaningful than to suggest that all cell lines in culture convert to one cell type or are overgrown by one type of contaminant.

The zymogram is apparently a stable characteristic of a particular cell line.<sup>17,18</sup> We have found that the zymogram patterns observed for each clone under each growth condition are stable through consecutive subcultures over a 2- to 6-month period. Recently we had occasion to determine the zymogram patterns from one of the clones that had been frozen for 2½ years. The patterns were identical to those observed previously. Other studies<sup>42,45-47</sup> as well as the work reported here indicate that the enzymic-specific activity is also a stable and reproducible characteristic of a particular cell line when determined at the same point in the growth cycle. The zymograms from individual clones grown in suspension culture can therefore be used to identify and distinguish the clones. When combined with studies to monitor specific enzymic activities, this procedure will allow for continual and rapid study of the effects of nutritional, environmental, and genetic changes upon the metabolic capability of cells in culture.

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Unclassified  
Security Classification

DOCUMENT CONTROL DATA - R & D		
(Security classification of title, body of abstract and indexing annotation must be entered when the overall report is classified)		
1. ORIGINATING ACTIVITY (Corporate author) Department of the Army Fort Detrick, Frederick, Maryland, 21701		2a. REPORT SECURITY CLASSIFICATION Unclassified
		2b. GROUP
3. REPORT TITLE IDENTIFICATION OF CLONES OF MAMMALIAN CELLS BY ENZYMIC ACTIVITY AND ISOENZYME DISTRIBUTION PATTERNS		
4. DESCRIPTIVE NOTES (Type of report and inclusive dates)		
5. AUTHOR(S) (First name, middle initial, last name) Donald C. Fish James P. Dobbs Richard C. Carter		
6. REPORT DATE July 1969	7a. TOTAL NO. OF PAGES 31	7b. NO. OF REFS 49
8a. CONTRACT OR GRANT NO.	8b. ORIGINATOR'S REPORT NUMBER(S) Technical Manuscript 472	
8. PROJECT NO. 1B562602A082		
c.	9b. OTHER REPORT NO(S) (Any other numbers that may be assigned this report)	
d.		
10. DISTRIBUTION STATEMENT Qualified requesters may obtain copies of this publication from DDC. Foreign announcement and dissemination of this publication by DDC is not authorized. Release or announcement to the public is not authorized.		
11. SUPPLEMENTARY NOTES		12. SPONSORING MILITARY ACTIVITY Department of the Army Fort Detrick, Frederick, Maryland, 21701
13. ABSTRACT Isoenzyme distribution patterns (zymograms) and enzymic-specific activities were determined for both suspension and monolayer cultures of the LM cell line, its clone (Cl 1), and four sub-clones from this clone. Identical zymograms were obtained from monolayer cultures of all clones for all enzymes tested. Suspension cultures of all clones yielded identical zymograms for six hydroxy acid dehydrogenases. However, when grown in suspension culture, reproducible and characteristic differences were found in the esterase zymograms from each clone. The growth pattern and esterase zymograms from sub-clone 1-9 varied more from those of the parent and the other sub-clones than any of the others. Thus, this procedure can be used to identify and characterize tissue cells and their clones in culture. The enzymic-specific activities from all cultures were identical when the cultures were sampled at the same stage in their growth cycle.		
14. Key Words *Clones Electrophoresis *Enzymes Genetics *Identifying *Tissue culture		